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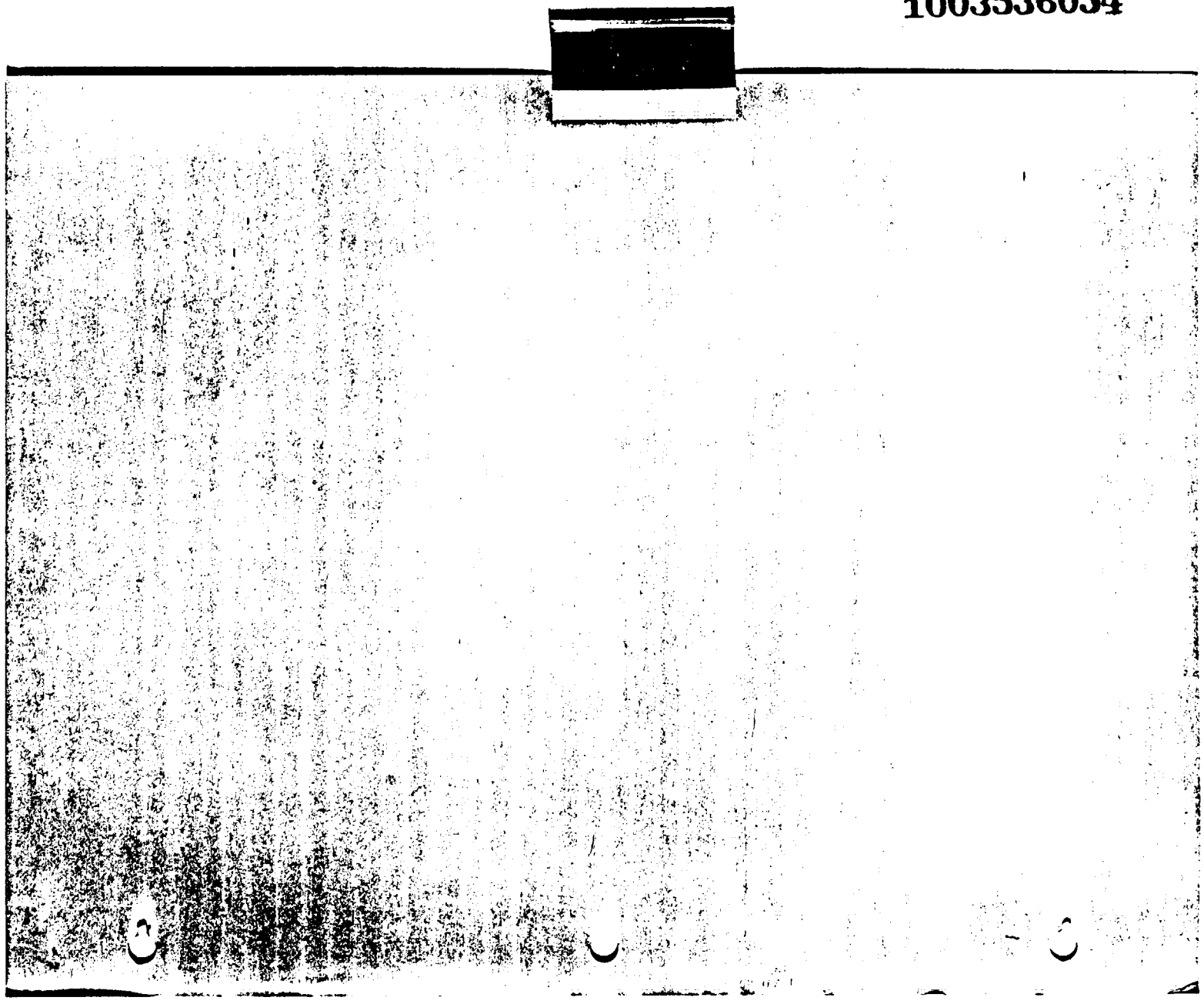

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Progress Report

from the period

November 1, 1974 through August 1, 1975

Contract No. 0025

from

The Council for Tobacco Research

"Human Aryl Hydrocarbon Hydroxylase Studies"

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Microbiological Associates August 15, 1975
Annual Report
CTR Contract 0025

I. Work accomplished, Milestones

A. Isolation of human lymphocytes.

Table 1 presents our step-by-step procedure for the isolation of human lymphocytes using ficoll-hypaque gradients. The ficoll-hypaque solution is now available from Microbiological Associates as a sterile working solution pre-tested to have a density of 1.08g/ml. Our normal yield of blood lymphocytes using this procedure is about 1.0×10^6 cells/ml whole blood. As measured by the Wrights stain, the isolated white blood cells are at least 90-95% small lymphocytes. Because of their rather uniform size, an automatic cell counter, such as the Fisher Autocytometer II, can be readily used to adjust the cell concentration to the required level.

B. Mitogen activation of human lymphocytes.

The reproducible activation of these isolated lymphocytes is the single biggest problem in the standardization of procedures designed to quantitate aryl hydrocarbon hydroxylase (AHH) activity in these cells. Figure 1 depicts the growth curve of mitogen activation of human lymphocytes when grown in 10 X 75mm test tubes. The optimal conditions are given in table 2. These are our "optimal" conditions; resulting in a 0.5-2 fold increase in cell number during the 96 hour incubation period. Activation is more efficient using 12% autologous serum and the 10 x 75mm test tubes, however, the 10-50% increase in activation observed, is more than offset by the physical impossibility of handling 2 culture tubes for every ml of whole blood. Therefore, the procedure of activation presently being done uses no autologous serum and 25 cm^2 tissue culture flasks.

The effects of various culture conditions on the mitogen activation of human lymphocytes are shown in tables 3-5. Table 3 demonstrates the effect of culture vessel, table 4 shows the effect of various lots of FCS, and table 5 depicts the effects of different sources and types PHA on lymphocyte growth in culture. The growth kinetics of human lymphocytes exposed to different concentrations of PHA-P are shown in figure 2. It is quite ob-

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vious that all of these parameters can definitely influence the number of cells responding to mitogen activation.

Another major problem which manifested itself during these studies is the relative difficulty of reproducibly counting activated lymphocytes. Figure 1 shows that the hemocytometer and autocytometer seem to reproducibly count cells for up to 72 hours of activation, but large discrepancies exist for 96 hour cultures. We believe the hemocytometer count to be more accurate because the cell counter cannot discriminate clumps of cells, dead cells, unactivated cells, or small cellular debris from intact lymphoblasts. Thus the hemocytometer is the method of choice for enumerating cultured lymphoblasts, but even this technique has definite shortcomings. The inherent error, even if 100-200 cells per 10 fields are counted, and great care is used, is about 10-15%. Moreover, the presence of clumps of cells, dead cells, and non-activated cells increases the error tremendously. The hemocytometer may be used if only 2-4 assays per week are attempted, but if 8-10 assays per day are required (which will definitely be the case), the hemocytometer is completely unworkable. For example, in order to assay 10 individuals (in triplicate) in one day, 120 separate hemocytometer counts would be required (10 people X 3 samples X 2 parameters (induced and non-induced) X 2 separate counts). To do this number of cell counts plus a careful assay in one day is barely feasible.

A possible alternative to cell counts is total DNA per culture. Figure 3 shows the growth curve and the DNA content of mitogen activated and non-activated human lymphocytes grown in Falcon 3013 flasks. The population of non-activated cells remains constant indicating no cell multiplication when measured either by cell number or total DNA (diphenylamine method). For mitogen-activated cells, DNA content and cell number parallel one another fairly well. Both show an initial drop before cell proliferation. Thus, DNA per culture gives an accurate measurement of the total viable cells per culture but not necessarily a measure of the degree of activation of the lymphocytes.

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C. Assay for AHH activity in mitogen-activated human lymphocytes.

With only minor modifications of our existing AHH assay system, we were able to detect and quantitate the enzyme activity associated with cultured human lymphocytes. The modifications were:

1) use of scintillation grade acetone and hexane as solvents,
2) use of deionized water of at least 18 mohm grade, and
3) use of Wheaton disposable 13 X 125 mm glass test tubes as assay vessels.

Under these conditions, the signal to noise ratio (MCA-induced fluorescence values per zero time control fluorescence values) is usually between 40 and 100. Using this modified assay, AHH activity can be detected in cultured human lymphocytes (table 6), the pH optimal for this activity is about 8.5 (table 7), activity is stable to freezing at -70° for at least 3 weeks (table 8), and enzyme activity in vitro is linear for at least 1.0 hour (figure 4). Table 9 shows AHH activity in lymphocytes from two individuals grown in the presence of PHA and PWM either alone or in combination. It is apparent that the two mitogens do not act synergistically nor are their effects additive. In figure 5, the effect of time of MCA induction on AHH activity is shown. Data suggest that the standard 24 hour induction period is less efficient in inducing AHH activity than treatment for 48, 72, or 96 hours. Highest activity is observed if MCA is added simultaneously with mitogens.

Of major importance is the fact that the inducibility ratios observed are not like those reported by Kellermann et al, nor those originally observed in our laboratory (that is, inducibilities of 2, 3 or 4 fold), but rather are in the order of 6 to 15 fold. Our new procedures allowing for optimal growth conditions must be the cause of these differences. The major reasons for these discrepancies are the very low constitutive (or non-induced) AHH activities now associated with these lymphocytes. We suggest that there is really no constitutive AHH activity in migoten-

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treated lymphocytes, but rather, the AHH level observed results from the non-specific response of these lymphocytes to contaminants in the culture medium. In this way, the lot-to-lot variations observed for FCS probably result from the presence of various levels of contaminating "inducers" of AHH activity. Data presented by Kellermann et al, and Paigen et al at the recent Orlando, Florida meetings tend to support this hypothesis and Kellermann feels that this "inducer" is time-labile and only relatively young pools of FCS contain this activity. However, we recently obtained the lot number of FCS from Paigen's group which gave inducibilities of 2, 3 and 4 fold in their laboratory. This serum, when tested in our laboratory with our own standard serum as a control, gave the results shown in table 10. The data indicate that with our method of culturing lymphocytes and assaying AHH activity, the two sera give about the same inducibilities, i.e. inducibilities between 6.0 and 9.0. The results, using the two activation procedures (using the 10 X 75mm test tubes which we described in earlier reports or the Falcon #3013 flasks as described by Gurtoo et al (Cancer Res. 35: 1235, 1975)) and a blind protocol are given in tables 11 and 12 respectively. Generally the data indicate that the assay yields reasonable duplicates for a blood sample from one individual if samples were taken and assayed on separate days. Therefore, our culture conditions which seem to result in reliable and efficient cell activation have resulted in rather poor constitutive AHH activity, and it necessarily follows, a rather irreproducible inducibility ratio.

We recently concluded a study of 9 standard males in our laboratory in which blood samples from each test individual were drawn, cultured, and assayed weekly for four consecutive weeks using the RPMI method. Table 13 gives the results, expressed as AHH units per flask, per mg DNA, and per 10^6 viable lymphocytes, for a representative individual. Variations exist no matter which parameter is used to express non-induced and induced activities.

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D. Use of frozen lymphocytes.

Table 14 demonstrates that human lymphocytes can be slow-frozen (using a step freezer) and stored at -70° for up to 2 weeks and subsequently assayed for AHH activity. The frozen cells can be thawed (with about a 50% drop in viability), mitogen-activated for 72 hours, induced with MCA for 24 hours, and then assayed for AHH activity. Results indicate that AHH activity can be detected in these frozen-mitogen-activated cells, but the level is different from freshly cultured blood. This difference may not mean anything because we observe these same differences between two freshly cultured samples. Of importance is the fact that lymphocytes from populations that may be unavailable at a later date can perhaps be taken and frozen now, and subsequently assayed at a later time.

E. Use of chemically-defined medium.

The problems with lot-to-lot variation with FCS could perhaps be circumvented by the use of serum-free medium. This approach has worked well with tissue culture cells (Higuchi and Robinson, In Vitro 9: 114, 1973). The AHH activity of human lymphocytes cultured in a chemically-defined medium is shown in table 15. Poor induced and non-induced activity is observed in these cultured cells even if the medium is supplemented with up to 5% FCS. Some activity, however, is observed, and culture conditions will probably require modification in order to effect good mitogen-activation.

F. Use of other reference standards for determination of AHH activity.

The utility of presenting the enzyme activity in terms of AHH units per 10^6 cells has its obvious problems. To devise an alternate reference standard, two requirements seem necessary: (a) that total cellular content be easily and reproducibly quantitated, and (b) that some method for the deduction of the degree of mitogen-activation be used. We are presently evaluating methods to fulfill these requirements. Total cellular con-

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tent is being measured by quantitation of total DNA, using the diphenylamine method. Degree of activation is measured by observing the total DNA synthesis (^{3}H-thymidine uptake) occurring during the 96 hour culture period or during the final 24 hours of the culture period. Both total DNA and total DNA synthesis can conveniently be measured on the same sample (table 16). Figure 6 gives data from a recent experiment and shows the relationship between AHH activity, total cell DNA, and ^{3}H-thymidine incorporation into DNA as a function culture time. It looks as if the level of MCA-induced AHH parallels the rate of DNA synthesis.

An alternate approach to expressing AHH activity may be the use of the rate of induction of enzyme activity. Figure 7 shows the rate of induction of AHH from two individuals. If this is reproducible, with each person possessing a characteristic induction curve, then the slope of such a curve, or the total area under the curve, may be valid methods of categorizing individuals.

From the study with 9 standard males (see section C and table 13) we reached the tentative conclusion that data might better be expressed in terms of the induced specific activity, rather than inducibility. When the inducibilities (average values of twelve determinations) are plotted as a distribution, one obtains the pattern shown in figure 8, and when induced specific activities are plotted, one obtains figure 9. There seems to be no difference between individuals if AHH activity is expressed as inducibilities. However, if expressed as just units of AHH activity per mg DNA, three groups are observed. The ranges of these specific activities would certainly overlap, nonetheless, it is interesting that some differences were observed. Moreover, individuals such as GG, TR, and HR, have historically been higher inducers.

G. Use of another assay system to measure mixed function oxidase activity in human lymphocytes.

Measurement of another P-450-dependent enzyme activity in cultured human lymphocytes, O-deethylation of ethoxy-

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resorufin is summarized in table 17. This assay could be a very feasible way to measure the level of MCA-induced microsomal enzyme activities because: (a) there seems to be only one end product of enzyme activity, resorufin; (b) the activity can be measured directly with no extraction steps, and (c) the assay would be amenable to automation. This work is being done in collaboration with Dr. M. C. Burke (Karolinska Institute, Stockholm, Sweden).

H. Use of levels of NADH-cytochrome C reductase as a parameter on which to base AHH activity.

In collaboration with Dr. R. Prough (Southwest Medical School, Dallas, Texas), another microsomal enzyme has been studied that could possibly be used as a parameter on which to base AHH activity. A summary of the properties of this enzyme is given in table 18. Of importance is the fact that the level of this enzyme seems dependent on the degree of mitogen activation (figure10), and not on MCA treatment. Thus, the level of NADH-cytochrome reductase may reflect the absolute amount of microsomes in mitogen-activated cells and could serve as a basis on which to compare AHH activities from separate individuals. This procedure would obviate the need for either counting viable cells or determining total DNA in cultured human lymphocytes.

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II. Work to be accomplished

A. Evaluation of culture conditions to give reproducible cell growth, including use of different lots of FCS, use of chemically defined medium, use of purified mitogens with reproducible activating properties, use of other inducers of constitutive AHH (e.g. estradiol or tryptamine), and use of other inducers of "induced" AHH (e.g. benz(a)anthracene, 5,6-benzoflavone, or diphenyloxazole).

B. Evaluation of methods to correct for day-to-day variations in lymphocyte activation by the use of isotope incorporation into cell macro-molecules.

C. Repeat assays of cells cultured in GIBCO FCS and attempt to determine why inducibilities we obtained differ from those found in Kellermann's and Paigen's laboratories.

D. Evaluation of O-deethylation assay as a method for determining MCA-induced mixed-function oxidase activity in human lymphocytes.

E. Evaluation of rates of NADH-cytochrome C reductase as a parameter on which to base AHH activity.

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Table 1

Procedures for the Isolation of Lymphocytes from Whole Human Blood

1. The area where blood is to be taken is washed with a sterile prepodyne swab (Clinipad Corporation, Stamford, Connecticut).

2. Venous blood is collected in sterile 150ml evacuated containers (McGaw Laboratories, Milledgeville, Georgia) in which 1,000 units of sodium heparin have been previously added.

3. The blood is diluted 20% with sterile HBSS supplemented with penicillin and streptomycin.

4. In 16 x 100 mm plastic tubes, 6 ml of a solution of ficoll-hypaque (s.g. 1.080) is added.

5. 9 ml aliquots of the diluted blood are added to the 6 ml gradients.

6. The ficoll-hypaque gradients are spun at 1500 rpm (590 xg) for 40 minutes.

7. The lymphocyte band (at the interface between the ficoll-hypaque and plasma) is collected with sterile Pasteur pipettes and transferred to 50 ml centrifuge tubes.

8. Cells are washed twice with 25 ml aliquots of RPMI 1640 and cells are counted in an Autocytometer II (Fisher Scientific, Pittsburgh, Pennsylvania) and adjusted to a concentration of 0.5×10^6 cells/ml.

9. The complete medium is RPMI 1640, supplemented with 15% fetal calf serum 0.025M HEPES, 50 units penicillin per ml, 50 μg streptomycin per ml, 1% phytohemagglutinin-M (Difco Laboratories, Detroit, Michigan), and 1% pokeweed mitogen (Grand Island Biological Company, Grand Island, New York).

10. One ml aliquots of cells are added to 10 x 75 mm plastic culture tubes, or 8 ml aliquots are added to Falcon #3013 flasks and in either case, incubation is carried out at 37°, 5% CO_2 - 95% air.

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Table 2

Optimal Conditions for Mitogen-Activating Human Lymphocytes.

1. Medium

   a. RPMI - 1640
   b. 15% FCS (Lot # 86629 or 86929) (MA)
   c. 12% autologous serum
   d. 0.025M HEPES
   e. 50 units penicillin/ml, 50µg streptomycin/ml
   f. 1% - PHA-M (Lot # K9268) Burroughs-Wellcome, Inc.
   g. 1% - PWM (Lot # A 045502) GIBCO

2. Vessels

   a. 10 x 75 mm plastic tubes (Falcon)
   or b. $25cm^2$ tissue culture flasks (Falcon)

3. Cell Concentration

   a. 0.5×10^6 cells/ml in a total of 1.0ml/tube
   b. 0.5×10^6 cells/ml in a total of 8.0ml/flask

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Table 4

Effect of various lots of FCS on mitogen activation of human lymphocytes.[a,b]

	Lot #	# cells/ml at 96 hours (X 10^6)	Relative Effects
1.	84929	1.40	.93
2.	84970	1.36	.97
3.	82757	1.30	.93
4.	86539	.88	.63
5.	86629	.80	.57

[a]Cells were seeded at 0.5 X 10^6 cells/ml in 12 X 75mm tubes.

[b]Medium was RPMI supplemented with the various FCS at a concentration of 20% (v/v).

[c]Source of lymphocytes was one individual and all assays were done on the same day.

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Table 5

Effects of various sources and types of PHA on mitogen-activation.[a]

Mitogen		# cells at 96 hours (X 10^6)	Relative Effect
1. Burroughs-Wellcome	PHA-M	.80	1.00
2. Difco	PHA-M	.65	.81
3. Burroughs-Wellcome	PHA-P[b]		
	1.25	.93	1.16
	2.50	1.04	1.30
	5.00	.16	0.20

[a]Cells seeded at 0.5 X 10^6 cells/ml in 12 X 75mm tubes. Cells were from one individual and all assays were done on the same day.

[b]μg PHA-P/ml culture medium.

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Table 6

AHH inducibilities from human lymphocytes grown under our "best conditions".

Subject	N[a]	I[b]	Inducibility
EP	.02[c]	.11	5.5
GH	.02	.29	14.5
TR	.04	.55	14.0
RK	.02	.12	6.0
RS	.02	.12	6.0
RM	.02	.13	6.5
CM	.02	.38	19.0
HR	.03	.24	8.0

[a]Noninduced activity.

[b]MCA-treated cells.

[c]Values given in terms of pMoles 3-OHBP per 10^6 cells per min at pH 8.5.

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Table 7.

Effect of pH of Incubation Buffer[a,b]

Source	7.8	8.1	8.3	8.5	8.7	9.0
DA (ind.)	0.046 (3.3)	0.069 (3.0)	0.096 (3.8)	0.100 (3.6)	0.108 (219)	0.092 (2.7)
(n.i.)	0.014	0.021	0.025	0.032	0.037	0.034
GG (ind.)	-	-	0.113 (3.7)	0.143 (3.9)	0.166 (3.6)	0.166 (4.0)
(n.i.)	-	-	0.030	0.037	0.046	0.410

[a]Values given in terms of pMoles 3-OH BP per 10^6 cells per min. The assay was run for 45 minutes at 37°C and each tube contained 4 X 10^6 lymphocytes.

[b]ind. = MCA treated cultures; n.i. = non-induced controls.

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Table 8

Effect of storage at -70°C of mitog en-activated, MCA induced cells on AHH activity.[a]

Source	Fresh		Frozen[b]		Frozen	
	N	I	N	I	N	I
SG (10.9)[c]	0.011	0.120	0.012	0.110	0.012	0.120
DA (5.6)	0.045	0.253	0.040	0.240	0.032	0.230
RK (8.7)	0.021	0.182	0.022	0.190	0.028	0.210
IS (11.5	0.060	0.691	0.056	0.676	—	—

[a]Data given in terms of pMoles 3-OHBP per 10^6 cells per min. at pH 8.5.

[b]Cells were stored at -70°C as a pellet for at least 48 hours before assay.

[c]Relative inducibility is given parenthetically.

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Table 9

Effect of PHA and PWM on AHH activity in lymphocytes

	Non-induced		Induced	
	1[a]	2[a]	1	2
PHA[b]	0.73[d]	0.75	24.0	11.0
PWM[c]	0.70	0.60	2.7	2.2
PHA + PWM	0.44	0.98	14.2	6.9

[a]Two individuals were assayed.

[b]PHA concentration was 1%

[c]PWM concentration was 1%

[d]Data given in terms of fluorescent units per flask; approximately 4 X 10^6 cells/flask.

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Table 10

AHH activity in lymphocytes grown in medium containing FCS from either Microbiological Associates or Grand Island Biologicals

	FCS Source					
	Micro			GIBCO		
Subject	N	I	Ind	N	I	Ind
MM	0.75[a]	4.6	6.1	1.0	5.2	5.2
SG	0.91	7.9	8.7	0.84	5.1	6.1

[a]Data given in terms of fluorescent units per flask. Approximately 4×10^6 cells assayed per flask.

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Table 11

Assay of AHH activity using blind protocol.

Subject	Date	AHH #	N[a]	I[b]	Inducibility
JA	10/9	103	.030[c]	.17	5.6
	10/9	107	.033	.14	4.3
	10/9	109	.025	.15	6.0
	10/9	111	.047	.21	4.5
EA	10/10	116	.051	.12	2.4
	10/10	117	.053	.16	3.0
	10/10	119	.027	.11	4.1
	10/10	121	.023	.09	3.9
	10/24	153	.053	.74	13.9
	10/24	154	.101	.91	9.0
	10/24	156	.071	.51	7.3
	10/24	158	.044	.53	12.0
MH	10/3	94	no growth	—	—
	10/3	96	no growth	—	—
	10/3	97	no growth	—	—
	10/3	98	no growth	—	—
	10/24	152	.05	.25	5.0
	10/24	155	.06	.33	5.5
	10/24	157	.07	.27	3.9
	10/24	159	.04	.26	6.5
	11/7	190	no growth	—	—
	11/7	191	no growth	—	—
	11/7	194	.05	.30	6.0
	11/7	195	.06	.28	4.7
MC	10/30	162	.19	.44	2.3
	10/30	163	.21	.47	2.3
	10/30	165	.14	.31	2.2
	10/30	167	.16	.39	2.4
RT	10/30	164	.09	.24	2.7
	10/30	166	.12	.36	3.0
	10/30	168	.13	.41	3.1
	10/30	169	.10	.27	2.7
MP	10/2	81	.16	.53	3.3
	10/2	82	.28	.57	2.0
	10/2	84	.15	.42	2.8
	10/2	86	.24	.48	2.0

[a]Noninduced activity.

[b]MCA-treated cells.

[c]Values given in terms of pMoles 3-OHBP per 10^6 cells per min at pH 8.5.

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Table 12

Assay of AHH activity using blind protocol and Roswell Park Procedure

Subject	Date	AHH#	N[a]	I[b]	Inducibility
RP	3/3	339	.65	4.4	7.54
	3/3	341	.75	6.1	8.15
	3/3	343	.73	4.2	5.75
	3/3	345	.60	4.9	8.17
RM	3/3	340	.26	1.52	5.87
	3/3	342	.35	1.55	4.43
	3/3	344	.36	1.25	3.48
	3/3	346	.35	1.25	3.58
LM	3/11	355	1.07	11.3	10.6
	3/11	356	1.33	10.0	7.5
	3/11	359	1.09	10.1	9.25
	3/11	360	1.22	11.8	9.65
	2/11	298	0.59	1.95	3.30
	2/11	299	0.33	0.98	2.96
	2/11	301	0.45	0.85	1.88
	2/11	302	0.51	0.81	1.58
MA	3/11	357	1.13	7.3	6.45
	3/11	358	1.02	9.75	9.55
	3/11	361	.766	6.5	8.5
	3/11	362	1.43	4.75	3.33
	2/11	300	0.40	0.46	1.15
	2/11	303	0.41	0.59	1.44
	2/11	304	0.30	0.37	1.23
SM	2/25	331	1.49	12.1	8.12
	2/25	332	1.56	11.15	7.18
	2/25	335	1.18	12.5	10.6
	2/25	336	1.19	9.25	7.8
MH	2/25	333	.79	1.75	2.3
	2/25	334	.60	.82	1.37
	2/25	337	.49	.785	1.6
	2/25	338	.62	2.64	4.25

[a] Noninduced activity.
[b] MCA-treated cells.
[c] In terms of fluorescent units

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Table 13

Inducibility of TR based on different parameters.[a]

Assay	# flasks[b]	units/flask			units/mg DNA			units/10^6 cell		
		N	I	I/N	N	I	I/N	N	I	I/N
191	1	.075	.928	12.3	1.20	17.0	14.2	.063	.864	13.7
	2	.187	2.38	12.7	0.94	12.2	13.0			
	3	.216	2.68	12.4	0.98	11.8	12.0			
195	1	.100	.91	9.1	2.0	20.5	10.2	.058	.640	11.0
	2	.160	1.95	12.2	1.30	17.0	13.1			
	3	.280	2.69	9.6	1.80	20.1	11.2			
199	1	.074	1.73	23.5	1.30	32.4	24.9	.030	.448	14.9
	2	.136	3.25	23.9	1.34	27.4	20.4			
	3	.174	4.56	26.1	1.08	29.8	27.6			
203	1	.075	.98	13.1	1.3	16.6	12.7	.238	2.21	9.3
	2	.172	1.82	10.6	1.4	14.4	10.3			
	3	.180	2.40	13.3	1.3	16.0	12.3			

[a] Data given as described in headings of columns.

[b] Number flasks pooled to do assay; and at least duplicate samples assayed.

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Table 14

Effects of freezing whole lymphocytes at -70°C prior to assay.[a]

Source	Fresh		Slow Frozen[b]	
	N	I	N	I
SG	0.029	0.081	0.046	0.097
HR	0.046	0.133	0.071	0.200
GG	0.060	0.211	0.064	0.166
RK	0.030	0.060	0.038	0.064
CF	0.060	0.220	0.074	0.130
KT	0.057	0.110	0.057	0.198

[a]Values given in terms of pMoles 3-OH BP per 10^6 cells per min. The assay was run for 45 min at pH8.5 and each tube contained 4×10^6 lymphocytes.

[b]Lymphocytes were isolatated, then slow-frozen; subsequently, the cells were thawed, mitogen-activated, induced and assayed.

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Source: https://www.industrydocuments.ucsf.edu/docs/jlyl0000

Table 15

The Effect of FBS on Induced and Noninduced AHH Activity of Cells Grown in CDM

Sample (MG)	Non-Induced[a]	Induced[a]	Induced/Non-Induced
0% FBS	.14	.39	2.78
1% FBS	.145	.29	2.07
5% FBS	.22	.69	3.13
Control[b]	.91	12.6	13.8

[a]Activity given in terms of fluorescent units per flask (Flask - Falcon Plastics #3013 containing 8mls of media originally seeded with 1.5 X 10^6 lymphocytes per ml.)

[b]Control=0.5 X 10^6 lymphocytes per ml in RPMI 1640 with 20% FBS.

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Source: https://www.industrydocuments.ucsf.edu/docs/jfyl0000

Table 16

DNA Procedure

1. Add 5 - 7 ml cold (4°C) 2% potassium acetate in 95% ethanol to each assay tube containing the assayed cells, pool, and small amount of acetone-hexane.

2. Sonicate with microtip until the precipitate is well dispersed (5 - 30 sec).

3. Centrifuge at 1300 rpm for 20 min at 5° in PR-2.

4. Discard supernatant fraction and drain tubes.

5. Add 1-2 ml of 1 N PCA (depending on DNA concentration) and hydrolyze for 30 min at 85-90°.

6. Centrifuge at 1300 rpm for 30 min at 5° in PR-2 to pellet protein.

7. Remove two 0.10 ml aliquots of supernatant fraction for determination of radioactive DNA. Place each in vial containing 5ml Aquasol and count by liquid scintillation.

8. Take an additional 0.5ml aliquot of the supernatant fraction and add 1 ml of diphenylamine reagent (400 ml glacial acetic acid, 6 ml conc. H_2SO_4 and 6 g diphenylamine) containing 0.1 ml of acetaldehyde (16 mg/ml) per 20 ml of diphenylamine reagent.

9. Make standards with calf thymus DNA at concentrations of 0, 6, 12, 25, 50, and 75 μg/ml.

10. Place in dark for 22 hrs and read absorbance at 600 nM against a reagent blank or a zero time AHH assay. The components of the pool contribute to the absorbance.

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Source: https://www.industrydocuments.ucsf.edu/docs/jlyl0000

Table 17

Summary of data on O-deethylation activity of human lymphocytes in culture

1. Mitogen-activated human lymphocytes will metabolize ethoxyresorufin to resorufin *in vitro*.

2. The assay can be followed directly in a cuvette by quantitating the amount of fluorescent products associated with the presence of resorufin as a function of time in culture at 25°.

3. The activity is pH dependent with an optimum at 8.5.

4. Enzyme activity is substrate dependent with optimal concentration observed to be greater than 0.002mM. A substrate concentration of 0.004mM was routinely used.

5. Activity is dependent on concentration of NADPH. The optimal concentration being 0.2mM.

6. Under these conditions, enzyme activity is linear for up to 30 min.

7. Enzyme activity is dependent upon cell concentration giving a roughly linear response using cell concentrations from 1×10^6 to 8×10^6 cells per assay.

8. Mitogen-activated methylcholanthrene-induced human lymphocyte cultures can be frozen at -90° for up to two weeks and enzyme activity can still be measured.

9. In a survey of nine different individuals, the enzyme activity is preferentially induced by treatment with 3-methylcholanthrene. Enzyme activity in non-induced or control cultures is very low and barely measurable, however, activity from methylcholanthrene-treated cells is easily measured in culture.

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Source: https://www.industrydocuments.ucsf.edu/docs/jlyl0000

Table 18

Summary of data on NADH dependent cytochrome c reductase activity of human lymphocytes in culture.

1. Cytochrome c reductase has absolute requirement for NADH.

2. Activity is directly proportioned to number of mitogen-activated cells assayed 4-7nMoles cytochrome c reduced/ min/10^6 cells.

3. The pH optimum is 7.7.

4. Mitochondrial inhibitors e.g. KCN or antimycin A have no effect on enzyme activity.

5. Antibody to NADH-cytochrome c b_5 reductase inhibits enzyme.

6. Antibody to NADPH-cytochrome c reductase has no effect.

7. Antibody to cytochrome b_5 inhibits enzyme.

8. Activity is similar for 4 different people and is similar regardless of MCA treatment.

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Source: https://www.industrydocuments.ucsf.edu/docs/jlyl0000

Fig. 1 Time dependent increase of human lymphocytes during mitogen activation; (●——●) cell counts made with hemocytometers; (○——○) cell counts made with Fisher Autocytometer II.

Fig. 2 Effect of various concentrations of PHA-P on lymphocyte activation in culture

●——● = 0μg/ml □——□ = 1.25μg/ml

○——○ = 2.5μg/ml ■——■ = 5.0μg/ml

Fig. 3 DNA content (- - - -) and number of live cells (—————) in mitogen activated (●) and non-activated (○) lymphocyte cultures. Cell counts were made with hemocytometers; total DNA was determined by the diphenylamine method.

Fig. 4 In vitro metabolism of BP as a function of time. Lymphocytes were mitogen-activated for 72 hrs. and subsequently induced with MCA for 24 hrs. The relative inducibilities are given parenthetically.

Fig. 5 Effect of time of MCA induction on AHH activity. Cultures were induced with MCA at 0, 24, 48 and 72 hrs. (indicated by arrows) and assayed at 24 hr intervals as shown.

Fig. 6 MCA-induced AHH activity (○), (^{3}H)thymidine incorporation into DNA (●), and total DNA by the diphenylamine method (△). Cells were induced with MCA and labeled with (^{3}H) thymidine 24 hrs prior to assay. AHH activity, ^{3}H labeled DNA, and total DNA were assayed in the same cells in duplicate.

Fig. 7 Rate of increase of MCA-induced AHH activity for two individuals. All cultures were induced with MCA at 0 hrs. and assayed at designated times.

Fig. 8 Relative inducibilities of 9 individuals assayed for AHH activity at 3 different times. Each plot represents the average of 12 determinations.

Fig. 9 Specific activity of AHH of 9 individuals assayed at 3 different times. Each plot represents the average of 12 determinations.

Fig. 10 NADH-cytochrome c reductase activity and ^{3}H thymidine incorporation into DNA in PHA and pokeweed mitogen-stimulated lymphocytes. Cells were induced (X) or not induced (●) with MCA at 0 time and labeled with ^{3}H thymidine every 24 hrs. At the end of each 24 hrs. period cells were assayed for cytochrome c reductase, DNA, and ^{3}H thymidine incorporation.

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Source: https://www.industrydocuments.ucsf.edu/docs/jlyl0000

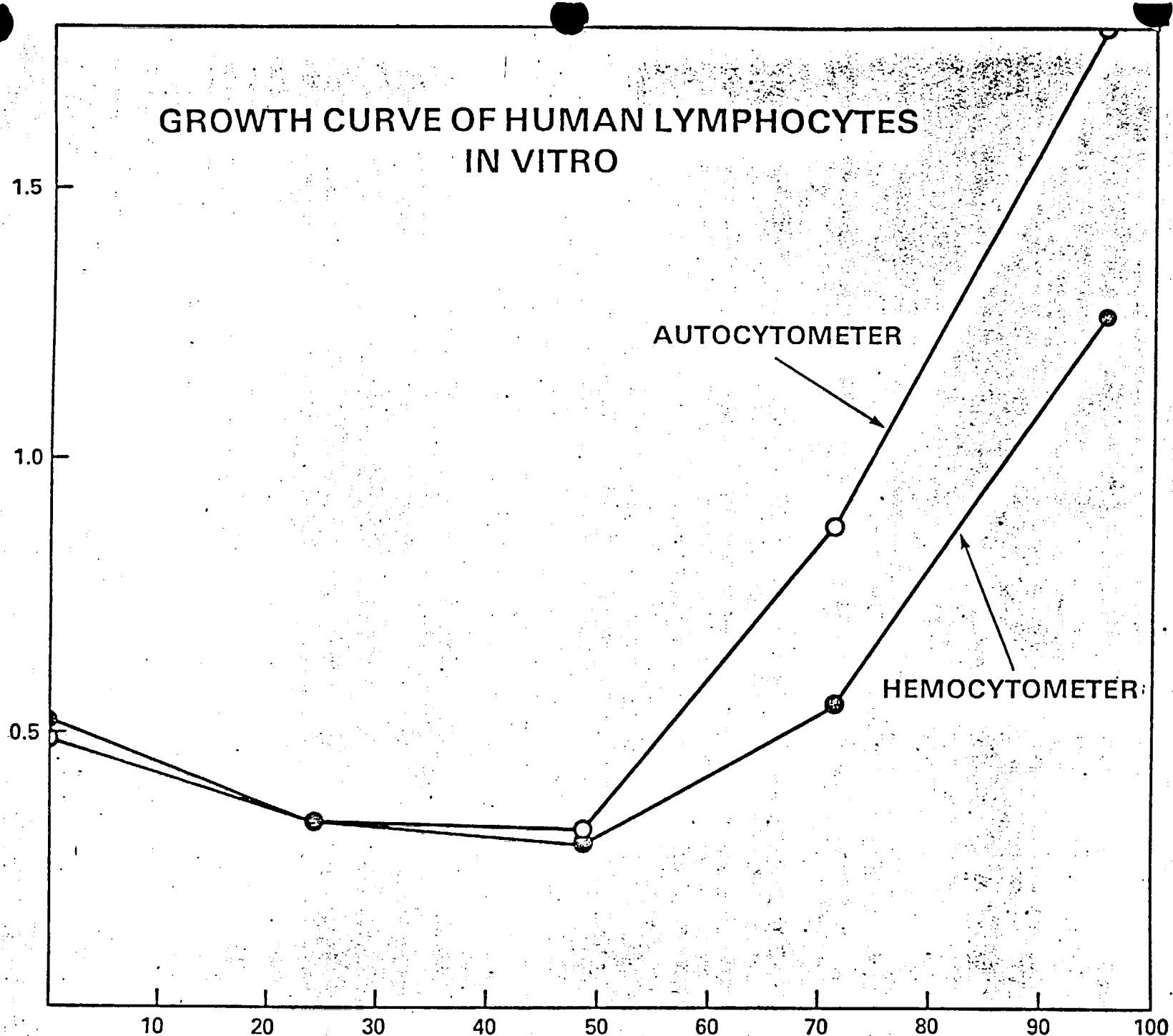

Figure 1

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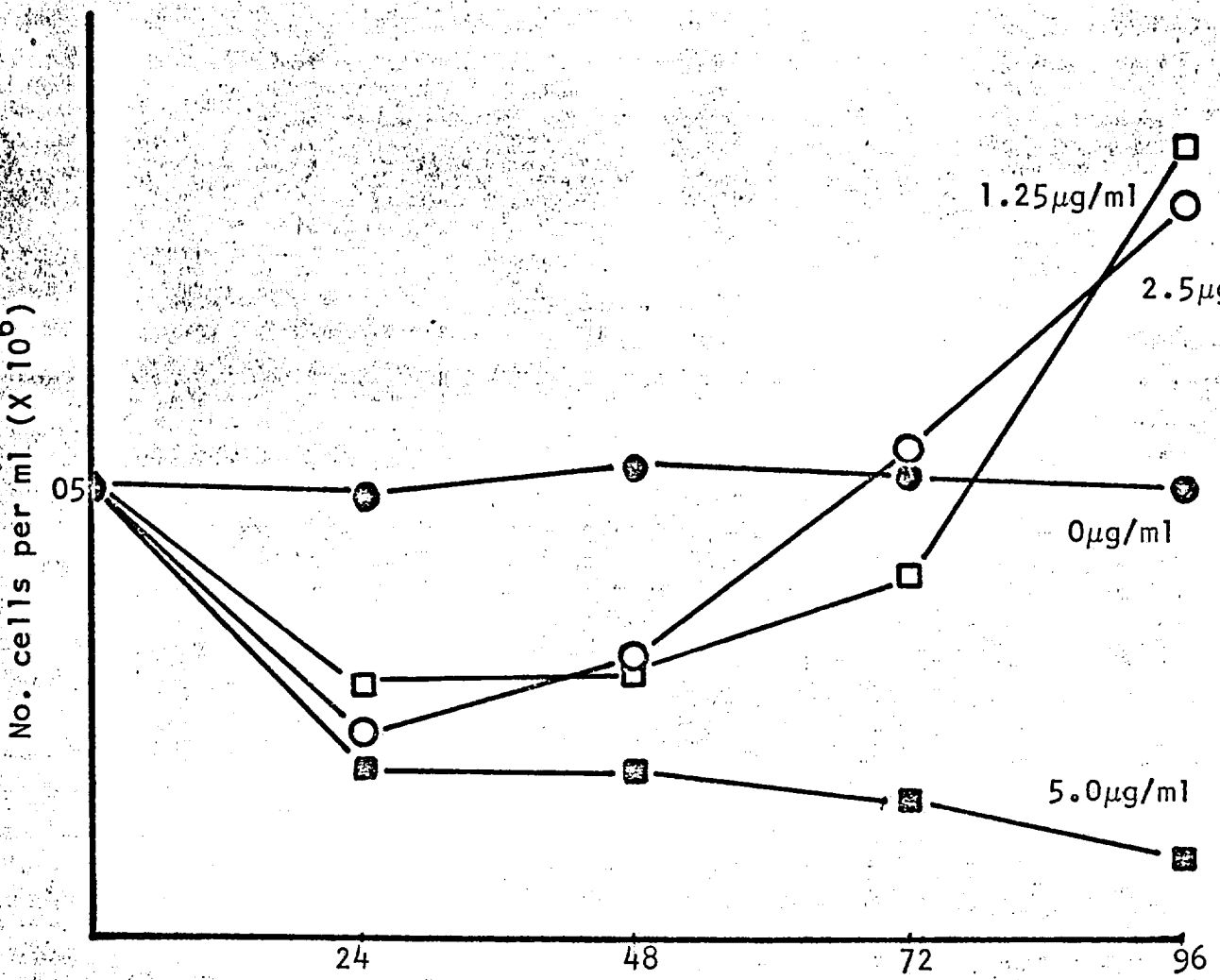
Figure 2



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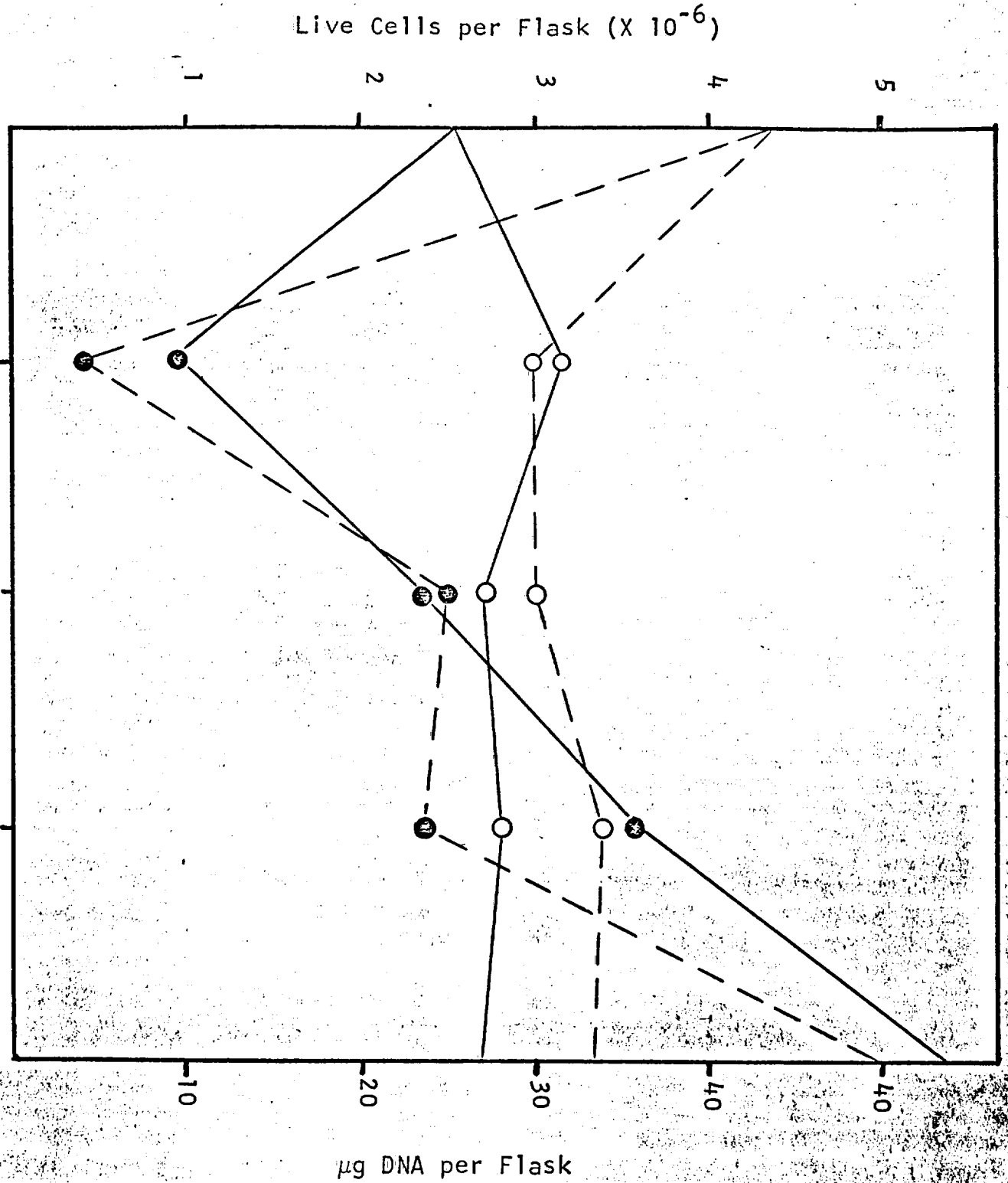

Figure 3

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Figure 4

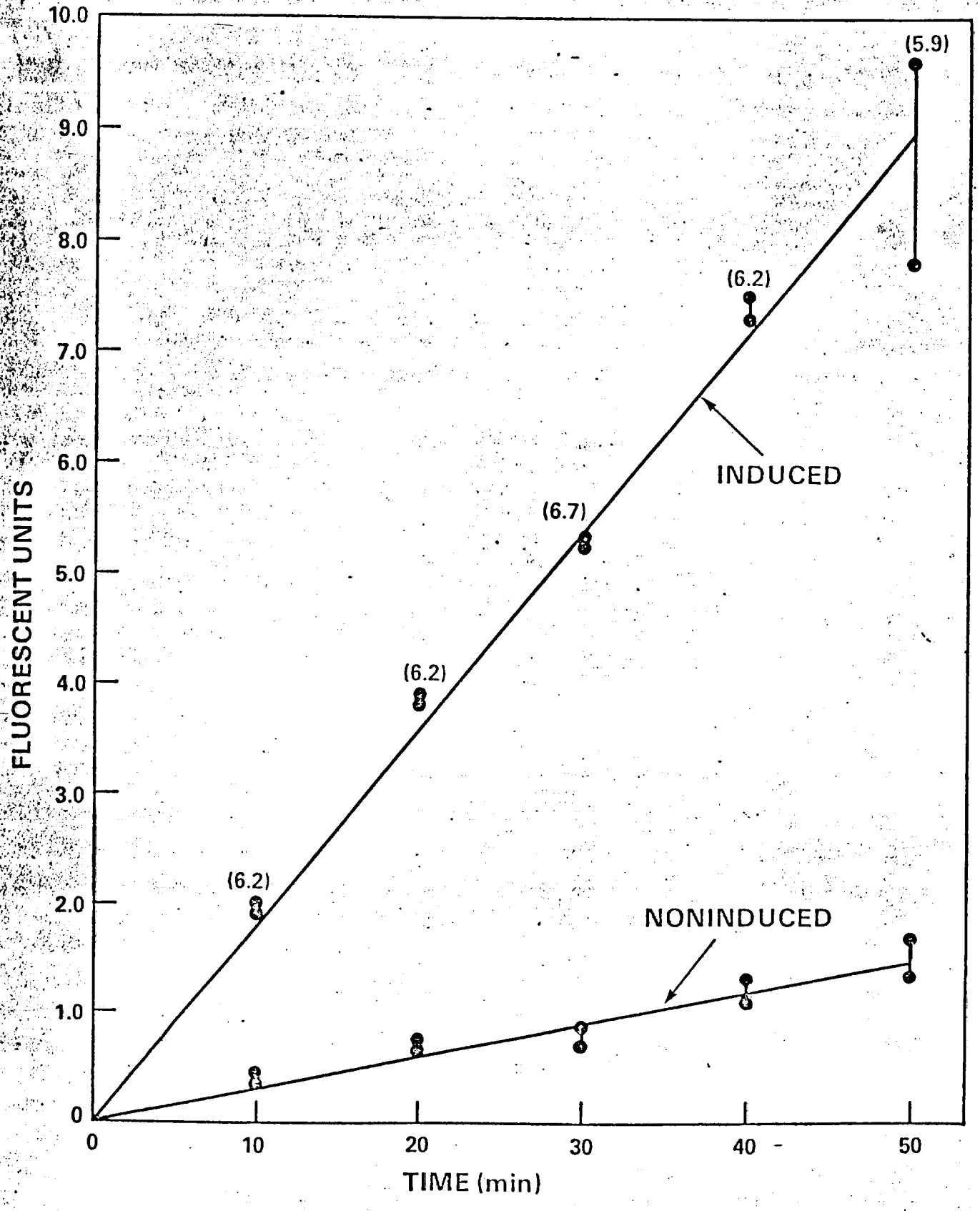

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Figure 5

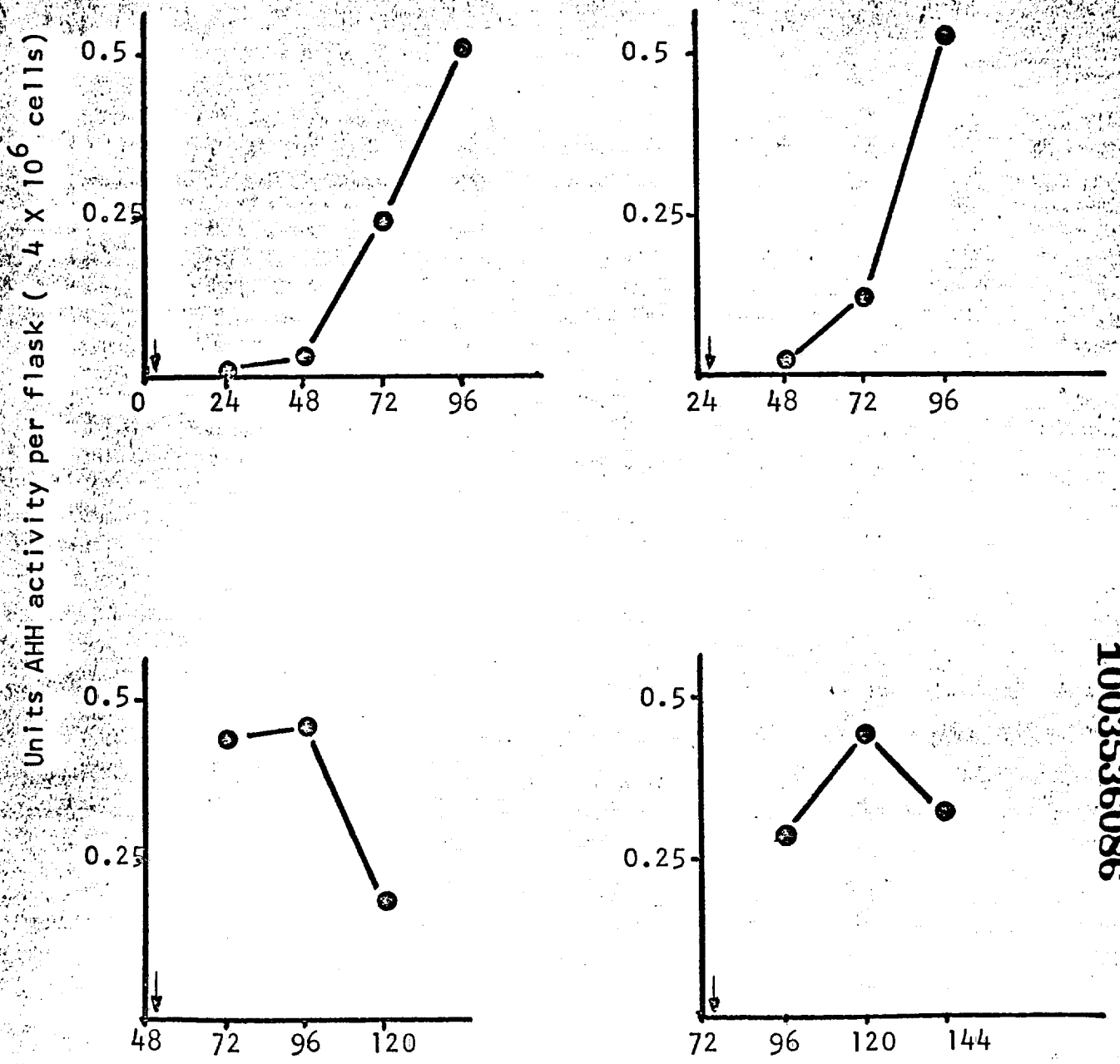

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Figure 6

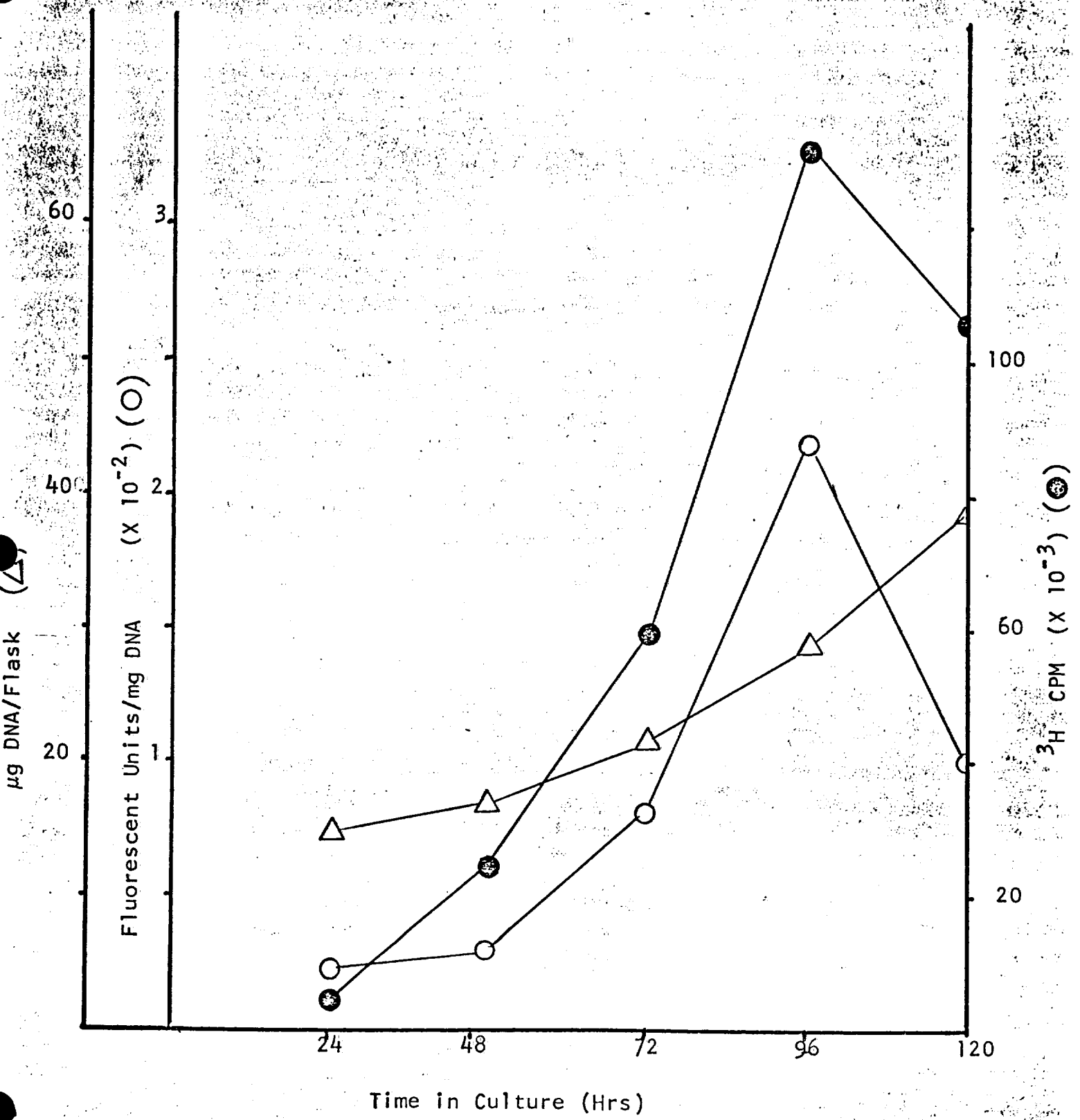

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Figure 7

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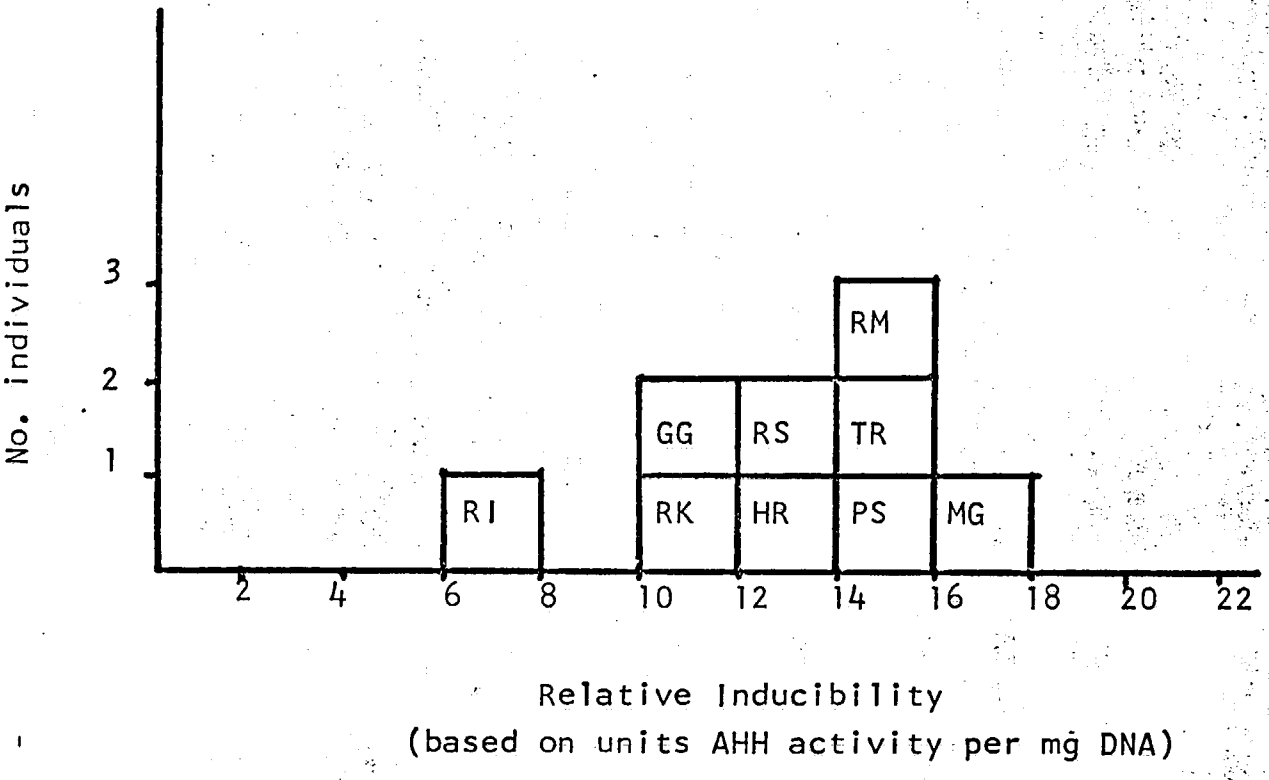

Figure 8

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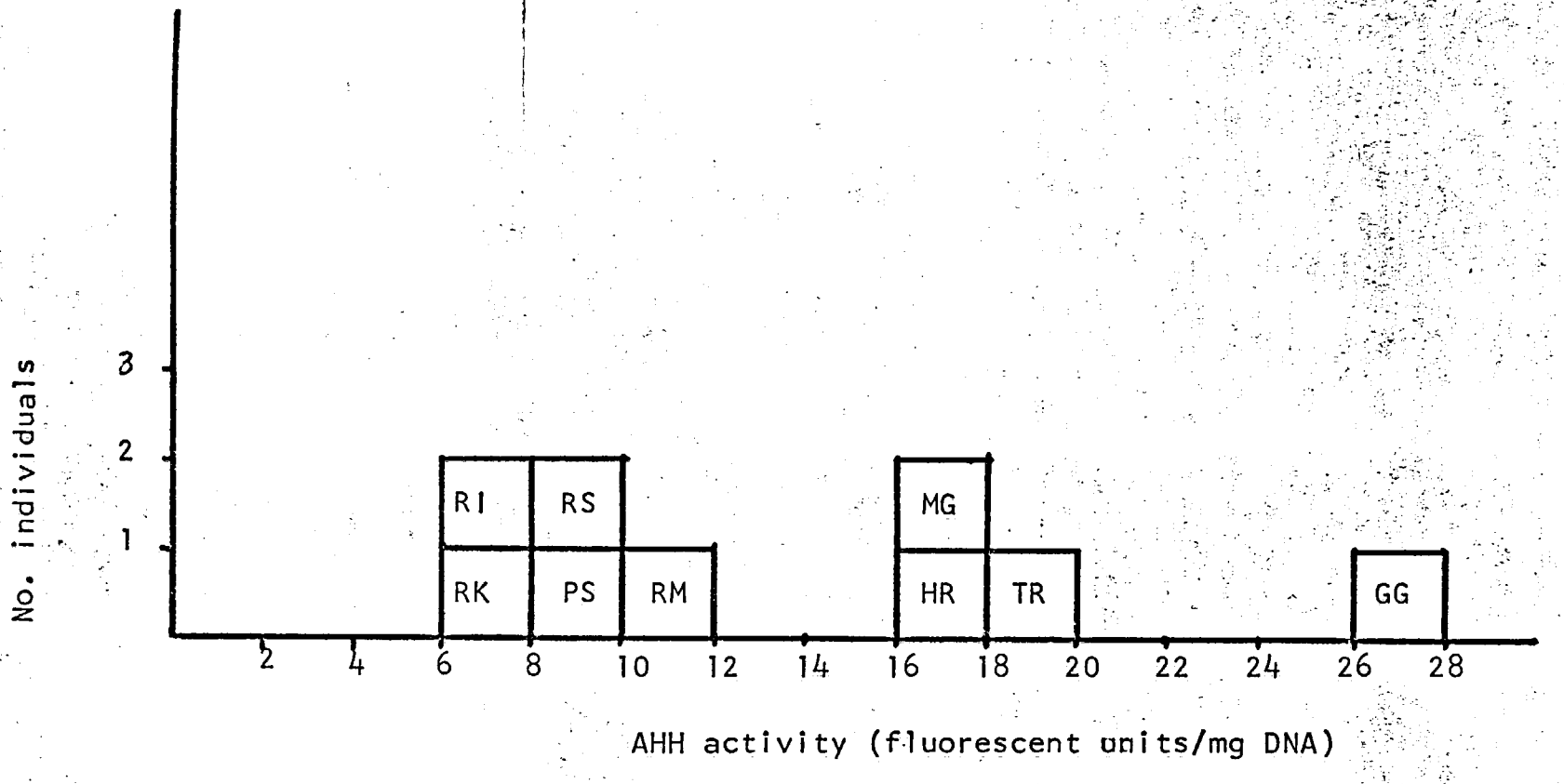

Figure 9.

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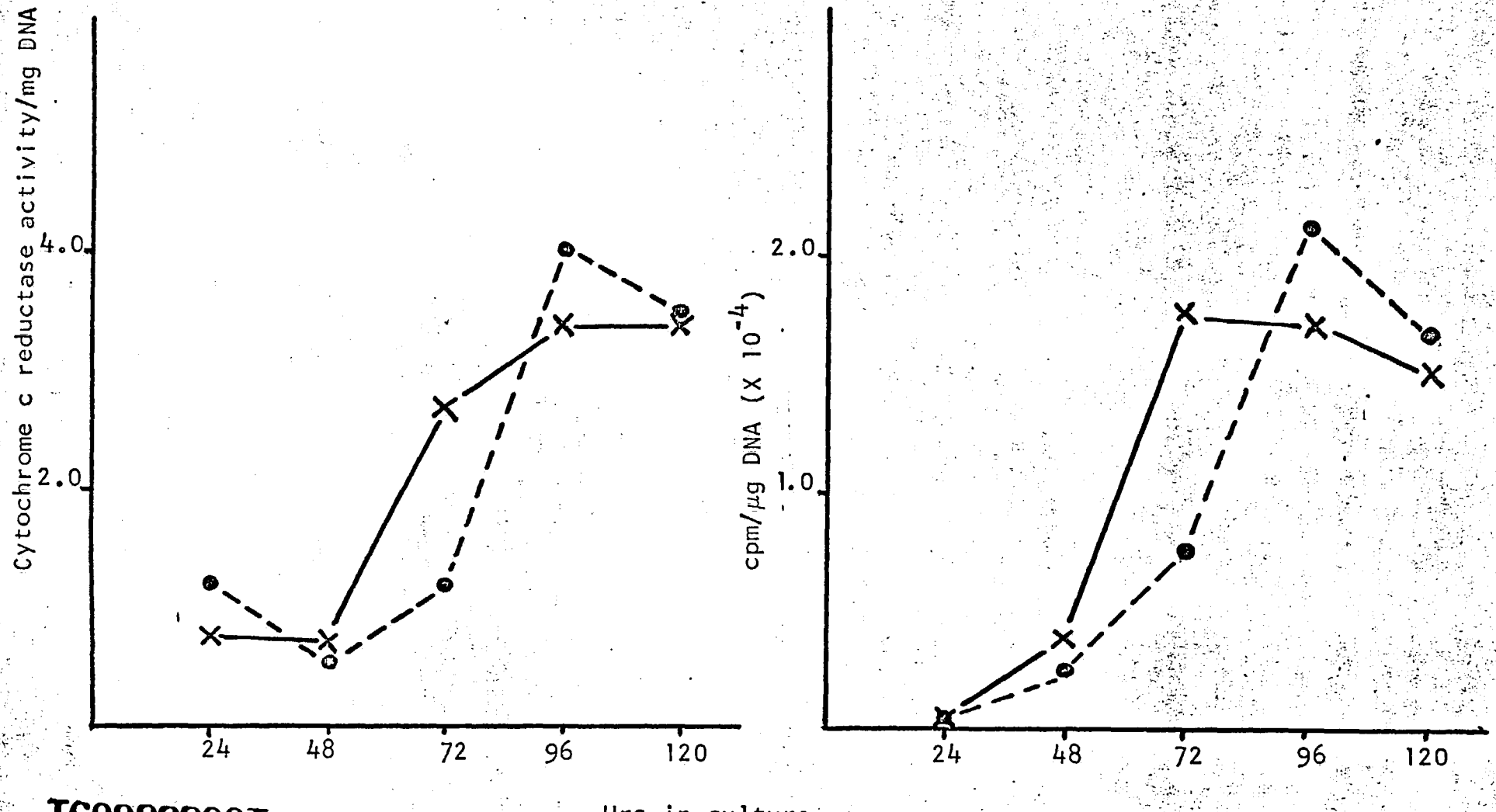

Figure 10

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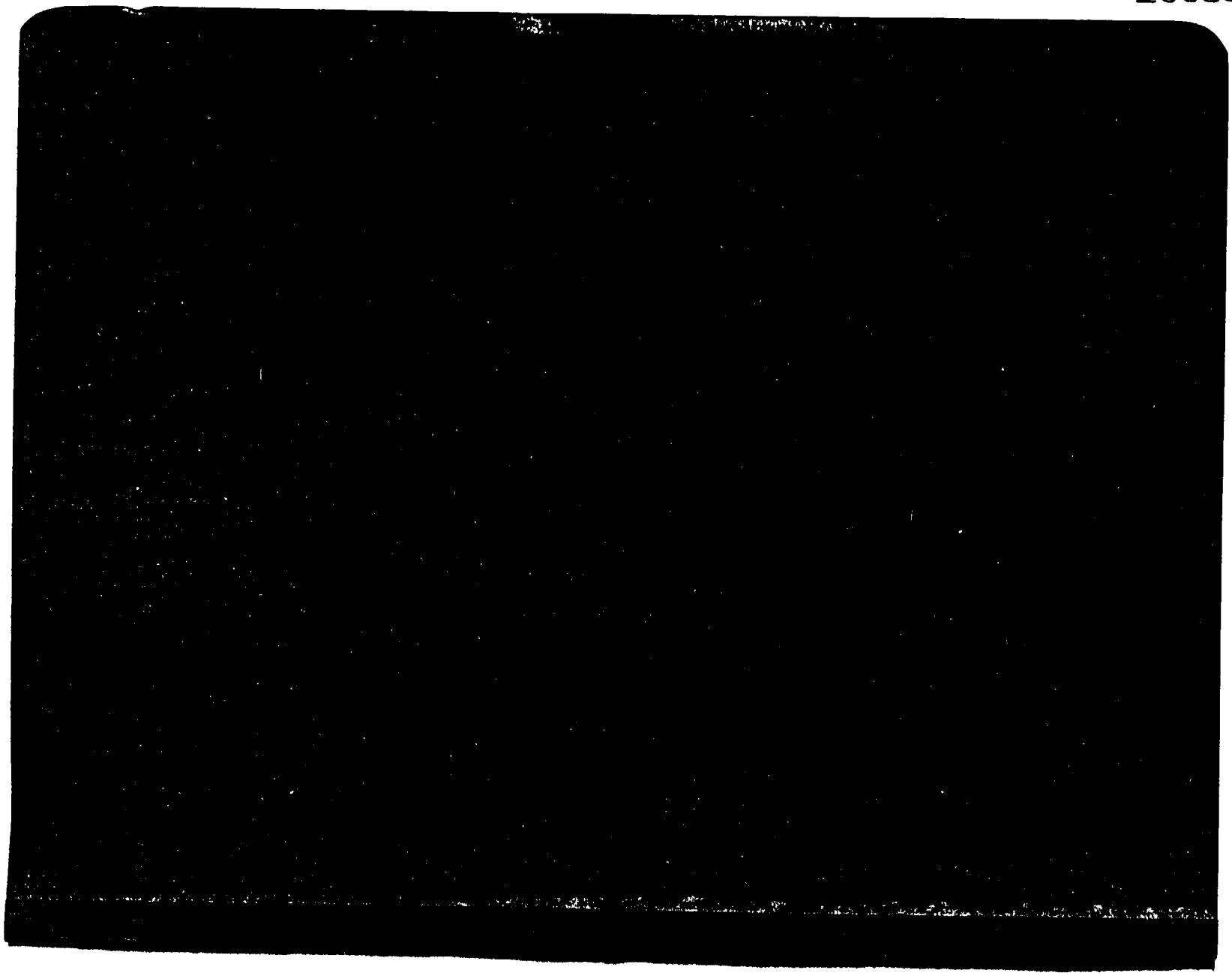

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